EXHIBIT A

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The Genetic Basis of Chronic Granulomatous Disease

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CHRONIC GRANULOMATOUS DISEASE

Phagocytic leukocytes (neutrophils, eosinophils, monocytes and macrophages) kill ingested micro-organisms by releasing microbicidal proteins from cytoplasmic granules and by generating superoxide (O_2^-) and other reactive oxygen species into the intracellular phagosomal compartment that contains the ingested micro-organisms (Fig. 1). The enzyme that catalyzes the formation of superoxide is an NADPH:O_2 oxidoreductase called NADPH oxidase. This enzyme is dormant in resting phagocytes and becomes activated upon adherence of micro-organisms to these cells. Reducing equivalents from NADPH are utilized to reduce molecular oxygen to O_2^-. In subsequent reactions, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and N-chloramines are formed, products that have increasing microbicidal potency and effective biological half-life.

If NADPH oxidase is dysfunctional, the phagocytes are unable to kill certain bacteria and fungi. As a result, patients with this disorder suffer from chronic granulomatous disease (CGD), characterized by severe recurrent bacterial and fungal infections of the subcutaneous tissues, the lungs and the lymph nodes, and occasionally the liver and the bones (Forrest et al. 1988). The most common pathogens include *Staphylococcus aureus*, *Aspergillus* species and a variety of gram-negative enteric bacilli including *Serratia marcecens*, *Pseudomonas cepacia* and various *Salmonella* species. CGD patients are particularly susceptible to organisms that contain catalase, because catalase prevents the CGD phagocyte from using microbial-generated H_2O_2 for killing these micro-organisms. Often chronic inflammations and multiple granulomas composed of giant cells and

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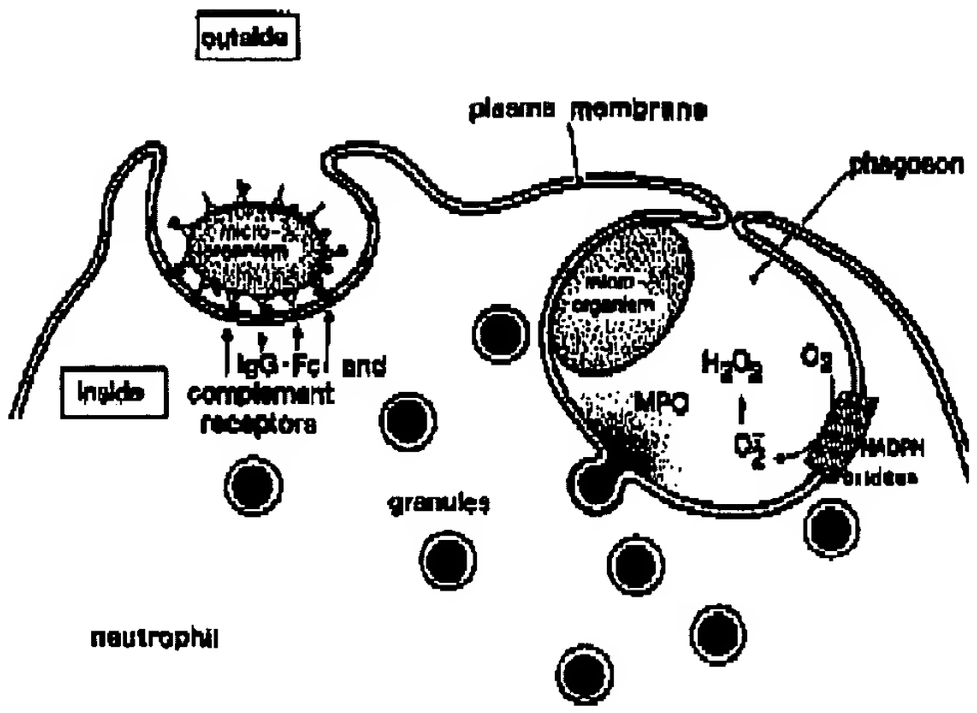

Figure 1. Schematic representation of phagocytosis, degranulation and generation of oxygen radicals. Micro-organisms opsonized with specific IgG antibodies and complement fragments C3b/iC3b (*) attach to Fc-gamma receptors and complement receptors, respectively. This attachment induces phagocytosis, fusion of intracellular granules with the phagosome membrane and activation of the NADPH oxidase. Superoxide generated by the NADPH oxidase is spontaneously dismuted into hydrogen peroxide (H_2O_2). One of the enzymes released into the phagosome is myeloperoxidase (MPO), which catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions. Reproduced from D. Roos (1991), with permission.

lipid-filled macrophages develop in CGD patients, which may obstruct gastrointestinal or urinary tracts. This feature has given its name to the disease. CGD is a rare disease, with an estimated incidence between 1:250 000 and 1:500 000. It usually manifests itself in early childhood and is predominantly found in boys. Due to increased knowledge about the composition, working mechanism and genetics of the NADPH oxidase, the clinical and genetic heterogeneity of CGD is now better understood. This has led to improved diagnosis and treatment of CGD patients.

NADPH OXIDASE

NADPH oxidase is a multi-component enzyme, consisting of at least five subunits. Two of these subunits are integral membrane proteins that together form the flavo-heme protein cytochrome b_{558}, the actual NADPH:O_2 oxidoreductase enzyme unit. The other three subunits are localized in the cytosol of resting phagocytes, translocate to cytochrome b_{558} in activated phagocytes and are probably needed to confer enzymic activity to cytochrome b_{558} by inducing a conformational change in the cytochrome. These three "cytosolic" subunits of NADPH oxidase

are a 47-kD protein called p47-*ph*
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assembled NADPH oxidase.

Cytochrome b_{558}

Cytochrome b_{558} is a heterodimer
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92 000, called gp91-*phox*. Each
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suggests that one heme is bound t
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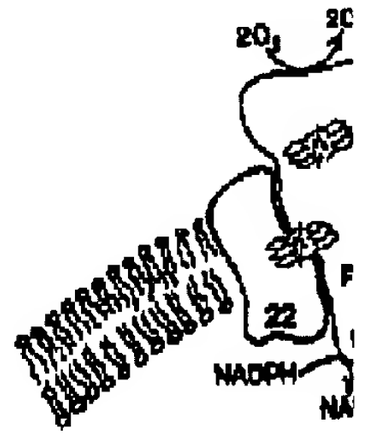

Figure 2. Schematic model of the
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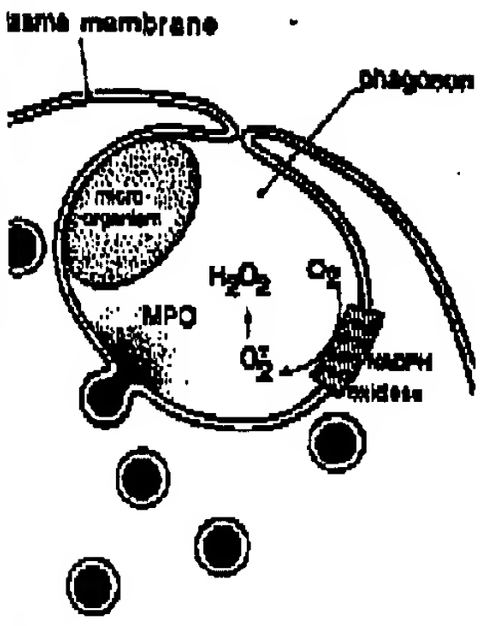

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are a 47-kD protein called p47-*phox* (p from protein and *phox* from *ph*agocyte *ox*idase) a 67-kD protein called p67-*phox* and a low molecular weight GTP-binding protein that may be either *rac*-1 (in macrophages) or *rac*-2 (in neutrophils). Together, these five proteins are sufficient to generate superoxide in a cell-free system consisting of recombinant proteins, NADPH, oxygen, GTP and an amphiphilic agent such as SDS or arachidonic acid to activate the oxidase (Rotrosen et al. 1993). In intact cells, however, additional proteins are probably involved in regulating the activation and deactivation of the NADPH oxidase (Abo et al. 1992, Mizuno et al. 1992, Kwong et al. 1993). Fig. 2 shows a model of the assembled NADPH oxidase.

Cytochrome b_{558}

Cytochrome b_{558} is a heterodimer consisting of a small alpha subunit with an Mr of 22 000, called p22-*phox*, and a larger beta subunit with an Mr of 76 000 to 92 000, called gp91-*phox*. Each cytochrome b_{558} molecule contains two heme moieties. The location of these heme groups is not known, but recent evidence suggests that one heme is bound to gp91-*phox* and the other one is shared between the two subunits (Quinn et al. 1992). Cytochrome b_{558} has a low redox potential

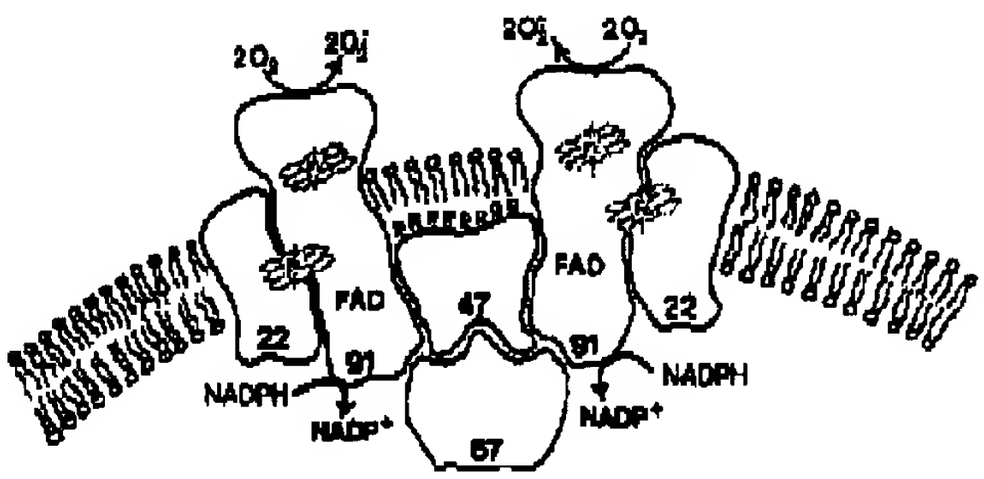

Figure 2. Schematic model of the phagocyte NADPH oxidase. In resting cells, p47-*phox* (47) and p67-*phox* (67) are located in the cytosol. After cell activation through ligand binding to plasma membrane receptors (see Fig. 1), p47-*phox* is phosphorylated, and p47-*phox* and p67-*phox* translocate to the membrane and integrate with the membrane-bound components gp91-*phox* (91) and p22-*phox* (22). Activating proteins (e.g. *rac*-2) also translocate to the membrane. This results in formation of an active NADPH oxidase complex, which accepts two electrons from each NADPH molecule at the NADPH binding site on gp91-*phox* and transmits these through FAD and the hemes to two molecules of oxygen at the other side of the membrane, thus generating superoxide $O_2^{\cdot-}$. According to Quinn et al. (1989) the model shows two cytochrome b_{558} molecules for each p47-*phox* and p67-*phox* molecule. Reproduced from D. Roos (1993), with permission.

and is therefore considered to be the NADPH oxidase component that donates electrons directly to molecular oxygen (Cross et al. 1981). Resonance Raman spectroscopy and electron paramagnetic resonance (EPR) data indicate that both heme groups contain a six-coordinate iron (Hurst et al. 1991, Isogai et al. 1993). This implies that oxygen cannot directly bind to the heme ion, but may instead be reduced to superoxide at the heme edge or at an extracellular site of the protein.

Recently, Segal et al. (1992) and other investigators (Rotrosen et al. 1992, Suminoto et al. 1992, Doussière et al. 1993, Taylor et al. 1993) found evidence for the existence of another prostetic group in cytochrome b_{558}, viz. FAD. This evidence was based (1) on sequence homology between the cytochrome b_{558} beta subunit and the NADPH and FAD binding regions of several mammalian, bacterial and plant flavoproteins, (2) on labeling of purified cytochrome b_{558} with an NADPH analogue, and (3) on the low FAD content of neutrophil membranes from cytochrome b_{558}-negative CGD patients (Bohler et al. 1986, Ohno et al. 1986). Thus, cytochrome b_{558} is probably a flavocytochrome that contains all necessary elements to accept electrons from NADPH at the cytosolic side of the protein and to donate these electrons to molecular oxygen at the extracellular (and intraphagosomal) side of the protein. Indeed, purified and relipidated cytochrome b_{558} is capable of generating superoxide without any additional proteins (Koshkin & Pick 1993).

Cytosolic components

In a cell-free NADPH oxidase activation system consisting of neutrophil membranes (containing cytochrome b_{558}), neutrophil cytosol fractions, GTP, NADPH and an amphiphilic agent (SDS or arachidonic acid), it has been found that the cytosol contains at least three proteins needed for superoxide generation by this system (Volpp et al. 1988, Nunoi et al. 1988, Bolscher et al. 1989). One of these proved to be a 47-kD protein (p47-*phox*) known to be phosphorylated in intact normal neutrophils after cell activation, but not in neutrophils from some CGD patients (Segal et al. 1985, Okamura et al, 1988, Bolscher et al. 1989). Later, this proved to be due to the absence of p47-*phox* in the phagocytes from these patients (Volpp et al. 1989). Two proteins have been cloned but, unfortunately, the amino-acid sequences of these proteins do not clarify their function. However, both p47-*phox* and p67-*phox* contain two regions that are 18–40% homologous with so-called SH3 regions of non-receptor tyrosine kinases, of which *src* is the classic example. Because such proteins move to the plasma membrane or cytoskeleton upon cell activation, these regions are supposed to be important for the binding of p47-*phox* and p67-*phox* to other cell proteins (e.g. cytochrome b_{558}).

The third cytosolic protein required for NADPH oxidase activity in the cell-free system has been called neutrophil cytosolic factor 3 (NCF-3) by Nunoi et

al. (1988), soluble oxidase componer
Sigma 1 by Pick et al. (1989). This
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Enzyme activation

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al. (1988), soluble oxidase component I (SOC-I) by us (Bolscher et al. 1989) and Sigma 1 by Pick et al. (1989). This protein needs GTP for its translocation to the plasma membrane (Bolscher et al. 1990, Philips et al. 1993). Recently, this protein has been identified as the low molecular weight GTP-binding protein *rac*-1 in macrophages (Abo et al. 1991) and *rac*-2 in neutrophils (Knaus et al. 1991, Mizuno et al. 1992). Subsequently, indications have been found for additional small proteins that regulate the GDP/GTP exchange of these *rac* proteins, and in this way may be involved in fine-tuning the activity of the NADPH oxidase activity (Abo et al. 1992, Mizuno et al. 1992, Kwong et al. 1993).

Enzyme activation

As indicated in the previous paragraphs, p47-*phox*, p67-*phox* and the *rac* proteins are supposed to be involved in the activation of NADPH oxidase upon attachment of opsonized micro-organisms to Fcγ and complement receptors on the phagocyte surface. Exactly how this process takes place is unknown, but the general idea is that p47-*phox* and p67-*phox* translocate from the cytosol to the plasma membrane and induce a conformational change in cytochrome b_{558}, thus allowing NADPH binding and/or electron flow from NADPH to oxygen. Indeed, translocation of p47-*phox* and p67-*phox* to the membrane has been observed both in intact cells and in the cell-free system (Ambruso et al. 1990, Clark et al. 1990, Tyagi et al. 1992, Park et al. 1992) and this process requires the presence of cytochrome b_{558} in the membrane (Clark et al. 1990, Heyworth et al. 1991). Peptides that mimic the cytosolic C-termini of the cytochrome b_{558} subunits inhibit this translocation and oxidase activation in the cell-free system (Rotrosen et al. 1990, Park et al. 1992, Kleinberg et al. 1992, Nakanishi et al. 1992). This does not necessarily imply that these regions are the actual docking sites of the cytosolic proteins, because high concentrations of these peptides were needed for efficient inhibition. In addition, we found that positively charged peptides in general inhibit this process (Verhoeven et al. 1993).

The translocation of p47-*phox* in intact cells is probably induced by the sequential phosphorylation of p47-*phox* at serine residues after activation of protein kinase C (Okamura et al. 1988, Heyworth et al. 1989, Rotrosen & Leto 1990). The translocation of p67-*phox* is dependent on the presence of p47-*phox*, but the reverse is not true (Heyworth et al. 1991, Uhlinger et al. 1993). Interaction between p47-*phox* and cytochrome b_{558} involves tyrosine-324 of p47-*phox* (Malech et al. 1993). This interaction is enhanced by diacylglycerol, whereas the translocation of p67-*phox* is enhanced by non-hydrolyzable analogues of GTP (Tyagi et al. 1992, Park & Babior 1993, Uhlinger et al. 1993).

The exact role of the *rac* proteins in this process remains to be established, but it is known that *rac* translocates to the membrane upon activation of intact cells (Quinn et al. 1993) or the cell-free system (Sawai et al. 1993). Post-trans-

lational processing of *rac*, e.g. removal of the C-terminal tripeptide, carboxylmethylation or prenylation, is needed for its interaction with GDP/GTP exchange-regulating proteins (Ando et al. 1992). Interaction with GDP dissociation stimulator (GDS) is needed for subsequent GTP binding and *rac* translocation (Takai et al. 1993) as well as NADPH oxidase activation (Ando et al. 1992, Heyworth et al. 1993). Possibly, *rac* translocation is needed for p67-*phox* translocation but not for p47-*phox* translocation.

Thus, the respiratory burst (sudden 30- to 100-fold increase in oxygen consumption and superoxide formation) in intact phagocytes may be initiated as follows. Ligand binding to surface receptors (e.g. Fc regions of opsonic immunoglobulins to Fcγ receptors, opsonic fragments of complement component C3 to complement receptors or high doses of chemotaxins to chemotaxin receptors) leads to a conformational change in these receptors and subsequent coupling of these receptors to tyrosine kinases or to membrane-bound trimeric GTP-binding proteins. In their turn, these proteins activate phospholipases and/or other protein kinases. This leads to formation of inositol phosphates and diacylglycerides, and to activation of low-molecular weight G-proteins. Thus, all necessary second messengers for oxidase activation are then present, and translocation of the cytosolic proteins may proceed.

Recent data suggest that p47-*phox*, p67-*phox* and *rac* translocate simultaneously in a 1:1:1 stoichiometry, possibly as a complex, to cytochrome b_{558} (Quinn et al. 1993). Exactly how p47-*phox* and p67-*phox* induce the NADPH oxidase activity is unknown. Cross & Curnutte (1993) found indications that p67-*phox* may be involved in permitting electron flow from NADPH to FAD in cytochrome b_{558}, whereas p47-*phox* may regulate electron flow from FAD to the heme moieties. Taylor et al. (1993) recently published a structural model of cytochrome b_{558} based on the known structure of ferredoxin-NADP reductase. In this model, the amino-acid sequence 413–503 in gp91-*phox* between alternating α helices and β sheets may, in the inactive state, prevent access of NADPH to the cleft that contains FAD. Activation, with access of NADPH to the FAD, could be induced by displacement of this sequence, possibly by direct binding of one or both of the cytosolic factors, following phosphorylation of the cytochrome upon oxidase activation (Garcia & Segal 1988).

Tissue specificity

Many cell types can generate superoxide, often in response to a specific stimulus. Of these cell types, phagocytes produce by far the largest amounts. Only EBV-transfected B-lymphocyte cell lines have been shown to contain the same NADPH oxidase as that found in phagocytes, because B-cell lines obtained from CGD patients show the same oxidase dysfunction as those found in the phagocytes from these patients (Volkman et al. 1984, Porter et al. 1992). For this reason, such cell lines are often used for im
oxidase components.

Fibroblasts contain another kind
potential cytochrome b_{558} (Meier et
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Fibroblasts contain another kind of oxidase, despite the presence of a low-potential cytochrome b_{558} (Meier et al. 1991, 1993). Reports on NADPH oxidase activity and immunoreactivity with antibodies against cytochrome b_{558} from human phagocytes in renal mesangial or glomerular cells (Radeke et al. 1991, Neale et al. 1993) await further characterization of the oxidase components in these cells.

Of the four "structural" components of the phagocyte NADPH oxidase, p22-*phox* is the only component with mRNA expression in cells other than phagocytes (Parkos et al. 1988). Attempts to identify the regulatory mechanisms of gp91-*phox* expression have been only partially successful. Skalnik et al. (1991a) have shown in transgenic mice that 450 base pairs of the 5′-flanking region of the gp91-*phox* gene are sufficient to cause expression of reporter genes in a subset of mononuclear phagocytes, but not in all myelomonocytic cells. In addition, the same investigators have identified a repressor region around the CCAAT box motif at about 160 and 170 base pairs 5′ from the gp91-*phox* initiation codon (Skalnik et al. 1991b) and a high-mobility group (HMG) chromosomal protein binding to this same region and supposedly acting as a transcriptional activator (Skalnik & Neufeld 1992).

CLASSIFICATION OF CGD

The two subunits of cytochrome b_{558}, p22-*phox* and gp91-*phox*, as well as p47-*phox* and p67-*phox*, have been cloned and their genes have been localized and characterized. Table I summarizes these data. Defects in any of these four NADPH oxidase components lead to absence of enzymic activity, and thus to development of CGD. An overview is given in Table II. Defects in other components, e.g. the *rac* proteins or GDP/GTP exchange proteins are not known, possibly because these proteins are involved in several essential cellular functions, and such defects may therefore be incompatible with life.

The alpha subunit of cytochrome b_{558} contains 195 amino acids (Parkos et al. 1988) with three or four hydrophobic regions that could serve as membrane-anchoring domains (Imajoh-Ohmi et al. 1992). The CYBA gene for this subunit is located on the long arm of chromosome 16 at 16q24 and contains six exons (Dinauer et al. 1990). Thus, mutations in this gene that inactivate p22-*phox* lead to an autosomal form of CGD (Dinauer et al. 1990). This type of CGD is rare, probably accounting for less than 10% of all CGD patients. Ten of these CGD patients from eight different families have been studied in detail (Table III).

The glycosylated beta subunit of cytochrome b_{558} (gp91-*phox*) contains 570 amino acids and appears as a smear of Mr 76 000 to 92 000 on SDS-PAGE. Five or six hydrophobic regions are present that could serve as transmembrane domains

TABLE I
Properties of NADHP oxidase components

		p22-*phox*	gp91-*phox*	p47-*phox*	p67-*phox*
Gene	Locus	CYBA	CYBB	NCF1	NCF2
	Chrom. location	16q24	Xp21.1	7q11.23	1q25
	Size	8.5 kb	30 kb	17-18 kb	40 kb
	Exons	6	13	9	16
mRNA	Size	0.8 kb	5 kb	1.4 kb	2.4 kb
Protein	Amino acids	195	570	390	526
	Mol. mass predicted	20.9 kDa	65 kDa	44.6 kDa	60.9 kDa
	Mol. mass SDS-PAGE	22 kDa	76-92 kDa	47 kDa	67 kDa
	pI	10.0	9.7	10	6
	Location in resting phagocyte	Membrane	Membrane	Cytoplasm	Cytoplasm
	Posttranslational modification	Phosphorylated	N-linked carbohydrates; Phosphorylated	Phosphorylated during oxidase activation	–

(Dinauer et al. 1987, Teahan et al. 1987). The CYBB gene for this subunit is located on the short arm of the X chromosome (Xq21.1) (Dinauer et al. 1987) and contains 13 exons (Skalnik et al. 1991b). Mutations in this gene account for all cases of X-linked CGD. This type of CGD is the most common one encountered, accounting for 50–60% of all CGD patients (Clark et al. 1989, Casimir et al. 1992). Table IV summarizes all mutations in X91 CGD patients known to me at the time of writing this review (November 1993).

Both subunits of cytochrome b_{558} are usually missing in A22 CGD as well as in X91 CGD (Verhoeven et al. 1989, Parkos et al. 1989). This indicates that single subunits have a decreased stability in comparison to the alpha-beta heterodimer. In a few cases, mutations in the alpha or beta subunit do not lead to absence of protein or heme, but only to loss of enzymic activity. These mutations may involve regions important for NADPH association or FAD binding to cytochrome b_{558} (Segal et al. 1992, Taylor et al. 1993). Occasionally, mutations are found that lead to partial loss of protein and heme. These mutations may involve regions important for heme binding and/or association of the two subunits. In analogy to the nomenclature used in describing thalassemia, these different phenotypes are now designated as $A22^0$ or $X91^0$ when no cytochrome b_{558} protein or heme is detectable (A = autosomal, X = X-chromosome linked), as $A22^-$ or $X91^-$ when subnormal amounts of cytochrome b_{558} protein or heme are detectable, and as $A22^+$ or $X91^+$ when normal amounts of cytochrome b_{558} protein or heme are detectable (see Table II).

TABLE II
Classification of CGD

Subtype	Frequency	Component Heme	gp91-*phox*	p22-*phox*	p47-*phox*	p67-*phox* protein	Defect in cell-free	Oxidase activity
$X91^0$	50–60%	–	–	–	–	–	+	–
$X91^-$	5–10%	+	+	+	+	+	+	–
$X91^+$	30–40%	+	+	+	+	+	+	+
$A22^0$	1–2%	–	–	–	–	–	+	–
$A22^-$	1–2%	+	+	+	+	+	+	–
$A22^+$	1–2%	+	+	+	+	+	+	+

LE I
P oxidase components

gp91-*phox*	p47-*phox*	p67-*phox*
CYBB	NCF1	NCF2
Xp21.1	7q11.23	1q25
30 kb	17-18 kb	40 kb
13	9	16
5 kb	1.4 kb	2.4 kb
570	390	526
65 kDa	44.6 kDa	60.9 kDa
76-92 kDa	47 kDa	67 kDa
9.7	10	6
Membrane	Cytoplasm	Cytoplasm
N-linked carbo-hydrates; Phosphorylated	Phosphoryl-ated during oxidase activ-ation	—

7). The CYBB gene for this subunit is mosome (Xq21.1) (Dinauer et al. 1987) b). Mutations in this gene account for all D is the most common one encountered, ients (Clark et al. 1989, Casimir et al. s in X91 CGD patients known to me at r 1993).

usually missing in A22 CGD as well as os et al. 1989). This indicates that single nparison to the alpha-beta heterodimer. beta subunit do not lead to absence of nzymic activity. These mutations may ociation or FAD binding to cytochrome Occasionally, mutations are found that . These mutations may involve regions ciation of the two subunits. In analogy halassemia, these different phenotypes no cytochrome b_{558} protein or heme is some linked), as A22$^-$ or X91$^-$ when protein or heme are detectable, and as f cytochrome b_{558} protein or heme are

TABLE II
Classification of CGD

Subtype of CGD	Component affected	Frequency (% of cases)	Heme spectrum	gp91-*phox* protein (blot)	p22-*phox* protein (blot)	p47-*phox* protein (blot)	p67-*phox* protein (blot)	Defect in cell-free system	Oxidase activity (% of normal)
X91^0	gp91-*phox*	~50	Absent	Absent	Trace	Normal	Normal	Membrane	0
X91$^-$	gp91-*phox*	5–10	Diminished	Diminished	Diminished	Normal	Normal	Membrane	10–30%
X91$^+$	gp91-*phox*	<5	Normal	Normal	Normal	Normal	Normal	Membrane	2–5%
A22^0	p22-*phox*	5–10	Absent	Absent	Absent	Normal	Normal	Membrane	2–3%
A22$^+$	p22-*phox*	<1	Normal	Normal	Normal	Normal	Normal	Membrane	0
A47^0	p47-*phox*	~30	Normal	Normal	Normal	Absent	Diminished	Cytosol	0–2%
A67^0	p67-*phox*	~5	Normal	Normal	Normal	Normal	Absent	Cytosol	0–2%

The cytosolic NADPH oxidase component p47-*phox* is composed of 390 amino acids (Volpp et al. 1989, Lomax et al. 1989). This protein is encoded by the NCF1 gene on the long arm of chromosome 7 at 7q11.23 (Francke et al. 1990a), which contains 9 exons spanning 18 kilobases (Chanock et al. 1991). Mutations in this gene found so far always lead to complete absence of the p47-*phox* protein, and thus to $A47^0$ CGD. Patients with this subtype of CGD comprise about 30% of all CGD patients.

Finally, the p67-*phox* protein contains 526 amino acids (Leto et al. 1990). The gene for his protein is NCF2, located on the long arm of chromosome 1 at position 1q25 (Francke et al. 1990a). This gene spans 40 kilobases and contains 16 exons (Kenney et al. 1993). Here, too, only $A67^0$ CGD patients are known. This CGD subtype is rare, accounting for less than 5% of all CGD patients.

Not only genetically but also clinically, CGD manifests as a very heterogeneous syndrome. This is apparent in the type of infectious micro-organisms, in the different infected tissues, in the frequency of the infectious episodes and in the age at which the patients present with the infections. This is understandable, given the heterogeneity in the molecular pathogenesis of the disease. We (Weening et al. 1985a) and others (Forrest et al. 1988, Margolis et al. 1990) have noted that, in general, patients with the cytochrome b_{558}-deficient forms of CGD follow a more severe clinical course than those with defects in cytosolic NADPH oxidase components. There is, however, no correlation between the amount of superoxide generated by the patients' phagocytes and the severity of the clinical course: patients with the $X91^-$ subtype of CGD, who may have neutrophils that generate 10–30% of the normal amount of O_2^-, suffer from infections as severe as patients without any NADPH oxidase capacity (Roos et al. 1992). In contrast, carriers of $X91^0$ CGD with only a few percent of normal neutrophils due to non-random X-chromosome inactivation may be completely healthy (Roos et al. 1986). Perhaps it is more beneficial to the host to possess a few neutrophils with full bactericidal capacity than to have a large number of neutrophils with low bactericidal capacity.

MUTATIONS IN THE ALPHA SUBUNIT OF CYTOCHROME b_{558}

Table III shows that all but 1 of the 8 A22 CGD patients had mRNA for p22-*phox* of apparently normal size in apparently normal amounts in their mononuclear leukocytes. In patient 1 without detectable mRNA for p22-*phox*, Southern blot analysis of genomic DNA revealed a homozygous deletion in the CYBA gene that removed all but the extreme 5' coding sequence of this gene (Dinauer et al. 1990). Patients 2, 3, 4, 5, and 6 were found to suffer from CGD due to point mutations in the open reading frame (Dinauer et al. 1990, de Boer et al. 1992a, Hossle et al. 1994). Patients 2 and 6 are compound heterozygotes for two mutations that predict a frameshift and a non-conservative amino-acid replacement.

TABLE III
Summary of p22-phox mutations in 10 patients with A22 CGD

Nr.	Patient	Sex	CGD type	Mutation type	Cytochrome b_{558} NADPH oxidase activity	Cytochrome b_{558} protein spectrum	mRNA p22-*phox*	Nucleotide change	Amino acid change	Reference
1.	L.N.	F	$A22^0$	deletion (homozygous)	0	0	N	> 10kb deletion	N.A.	Dinauer et al. 1990
2.	G.S.	M	$A22^0$	1) deletion 2) missense	0	0	N	1) C-272 deletion 2) G-297→A	1) frameshift 2) Arg-90→Gln	Dinauer et al. 1990

nent p47-*phox* is composed of 390 amino 9). This protein is encoded by the NCF1 at 7q11.23 (Francke et al. 1990a), which Chanock et al. 1991). Mutations in this te absence of the p47-*phox* protein, and ibtype of CGD comprise about 30% of

526 amino acids (Leto et al. 1990). The on the long arm of chromosome 1 at is gene spans 40 kilobases and contains), only A67⁰ CGD patients are known. r less than 5% of all CGD patients. CGD manifests as a very heterogeneous of infectious micro-organisms, in the y of the infectious episodes and in the the infections. This is understandable, athogenesis of the disease. We (Weening 988, Margolis et al. 1990) have noted ome b_{558}-deficient forms of CGD follow ith defects in cytosolic NADPH oxidase tion between the amount of superoxide nd the severity of the clinical course: who may have neutrophils that generate ver from infections as severe as patients Roos et al. 1992). In contrast, carriers normal neutrophils due to non-random pletely healthy (Roos et al. 1986). Per- o possess a few neutrophils with full umber of neutrophils with low bacteri-

BUNIT OF CYTOCHROME b_{558}

CGD patients had mRNA for p22-*phox* normal amounts in their mononuclear e mRNA for p22-*phox*, Southern blot mozygous deletion in the CYBA gene g sequence of this gene (Dinauer et al. nd to suffer from CGD due to point auer et al. 1990, de Boer et al. 1992a, mpound heterozygotes for two muta- conservative amino-acid replacement.

TABLE III

Summary of p22-phox mutations in 10 patients with A22 CGD

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA p22-*phox*	Nucleotide change	Amino acid change	Reference
1.	L.N.	F	A22⁰	deletion (homozygous)	0	0	0	N	>10kb deletion	N.A.	Dinauer et al. 1990
2.	G.S.	M	A22⁰	1) deletion 2) missense	0	0	0	N	1) C-272 deletion 2) G-297→A	1) frameshift 2) Arg-90→Gln	Dinauer et al. 1990
3.	O.P.	F	A22⁰	missense (homozygous)	0	0	0	N	C-382→A	Ser-118→Arg	Dinauer et al. 1990
4.	fam. S.	2F 1M	A22⁰	missense (homozygous)	0	0	0	N	G-297→A	Arg-90→Gln	De Boer et al. 1992a
5.	A.G.	F	A22⁰	missense (homozygous)	0	0	0	N	A-309→G	His-94→Arg	De Boer et al. 1992a
6.	S.B.	M	A22⁰	1) missense 2) insertion	0	0	0	N	1) A-186→G 2) insert G between C-194 and A-200	1) Glu-53→Val 2) frameshift, chain elongation, stop at codon 211	Hossle et al. 1994
7.	W.d.S.	M	A22⁰	splice/deletion (homozygous)	0	0	0	N	splice gtga→atga at start of intron IV	deletion exon 4	De Boer et al. 1992a
8.	I.L.	F	A22⁻	missense	0	N	N	N	C-495→A	Pro-156→Gln	Dinauer et al. 1991

0, zero; N, normal; N.A., not applicable. Patients 4 are two sisters and one brother. Patients printed in bold were analyzed in our laboratory (CLB, Amsterdam).

The same mutation leading to an Arg-90→Gln replacement in patient 2 is present in homozygous form in 3 patients from one family (nr. 4 in Table III). Patients 3 and 5 are homozygous for other missense mutations, resulting in other non-conservative amino-acid changes.

Patient 7 (Table III) is homozygous for a deletion of exon 4 in the p22-*phox* mRNA (de Boer et al. 1992a). PCR-amplified genomic DNA of this region had a normal size, indicating that the absence of exon 4 was not due to a deletion in the CYBA gene. The flanking intron sequence of exon 4 revealed a single point mutation in the consensus donor splice site sequence. Thus, in this patient, an mRNA splicing defect leads to skipping of exon 4. Because this is an in-frame deletion, a shortened polypeptide is predicted to be synthesized.

Patient 8 (Table II) is a homozygote for a mutation that leads to cytochrome b_{558} inactivation, but not to loss of cytochrome b_{558} protein or heme (Dinauer et al. 1991). Thus, this patient suffers from A22$^+$ CGD. The Pro-156→Gln substitution found in this patient was shown to occur in a cytoplasmic region of p22-*phox*. Perhaps this amino-acid substitution interferes with the interaction of cytochrome b_{558} with p47-*phox*, and in this way causes failure of NADPH oxidase activation (Nakanishi et al. 1992).

Fig. 3 shows a simplified structure of the alpha subunit of cytochrome b_{558} and the missense mutations in this polypeptide found so far. Mutations in the

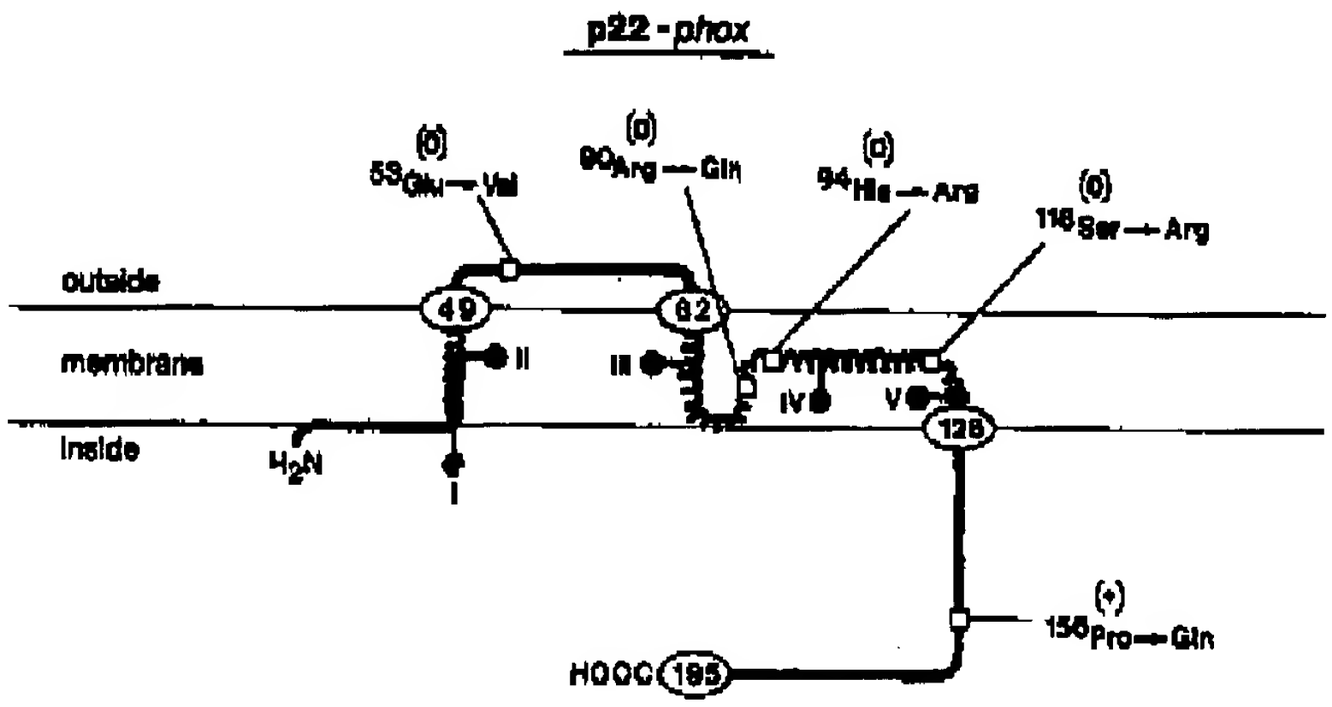


Figure 3. Schematic representation of p22-*phox*. Indicated are the possible orientation of the peptide in the membrane (Imajoh-Ohmi et al. 1992), the N- and C-terminus, the intron positions (roman numerals) and the missense mutations in the A22 CGD patients: (o) indicates A22^0, (+) A22$^+$ CGD.

N-terminal, hydrophobic half of t
expression. Apparently, such muta
phox protein or in p22-*phox* that
gp91-*phox*. Of special interest is th
III), which removes the histidine
(Dinauer et al. 1990, Quinn et al.
dines, His-72 is polymorphic and
for NADPH oxidase activity (Din
phils from patient 5 did not cont
Western blot, the His-94 substitu
association of p22-*phox* with gp91
the Pro-156→Gln mutation in
(patient 8, Table III) leaves the h

Altogether, nine different mu
families, indicating that this type
over, only four polymorphisms ha
phox so far (Dinauer et al. 1990,
in the structure of p22-*phox* alrea
this polypeptide.

MUTATIONS IN THE B

Deletions

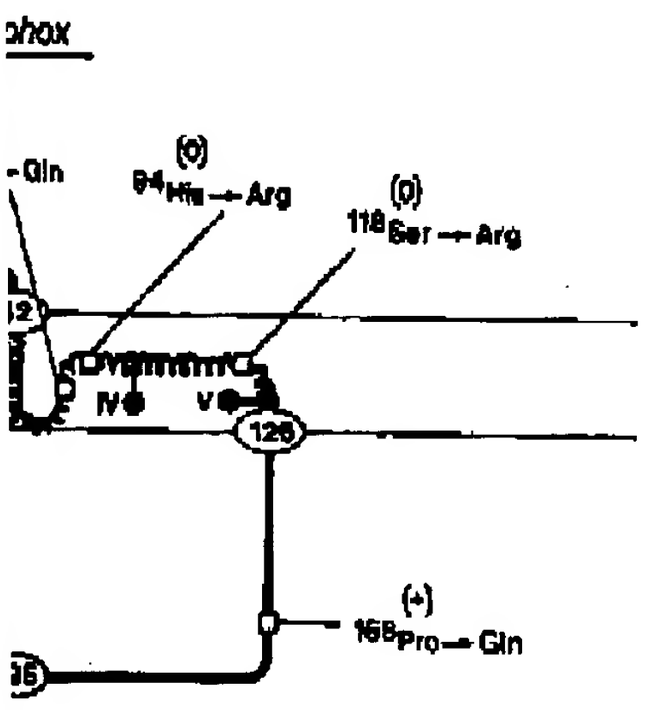
The first 12 patients shown in
deletion in the CYBB gene for
varies widely, from about 5000 b
with only one exception to the
the deletions are very large, not
genes as well. As a result, such
addition to CGD, e.g. Duchenn
McLeods's syndrome (a mild l
antigens due to defects in the re
(Table IV) (Kousseff 1981, Fran
et al. 1988).

Partial CYBB gene deletions
5–8, Table IV). These include tw
8.1 and 8.2), leading to deletio
Analysis of their genomic DNA
analysis of the PCR-amplified
very small overlap of the two d
lished). Remarkably, the mother

)→Gln replacement in patient 2 is present
one family (nr. 4 in Table III). Patients
sense mutations, resulting in other non-

for a deletion of exon 4 in the p22-*phox*
plified genomic DNA of this region had
e of exon 4 was not due to a deletion in
quence of exon 4 revealed a single point
site sequence. Thus, in this patient, an
of exon 4. Because this is an in-frame
licted to be synthesized.
for a mutation that leads to cytochrome
rome b_{558} protein or heme (Dinauer et al.
^{0} CGD. The Pro-156→Gln substitution
ar in a cytoplasmic region of p22-*phox*.
rferes with the interaction of cytochrome
s failure of NADPH oxidase activation

f the alpha subunit of cytochrome b_{558}
peptide found so far. Mutations in the



c. Indicated are the possible orientation of
al. 1992), the N- and C-terminus, the intron
mutations in the A22 CGD patients: (o)

N-terminal, hydrophobic half of the protein all result in loss of cytochrome b_{558} expression. Apparently, such mutations either result in intrinsically unstable p22-*phox* protein or in p22-*phox* that is unable to form a stable heterodimer with gp91-*phox*. Of special interest is the His-94→Arg substitution in patient 5 (Table III), which removes the histidine that is probably involved in heme binding (Dinauer et al. 1990, Quinn et al. 1992). Although p22-*phox* contains two histidines, His-72 is polymorphic and may be replaced by Tyr without consequences for NADPH oxidase activity (Dinauer et al. 1990). However, because the neutrophils from patient 5 did not contain measurable amounts of cytochrome b_{558} on Western blot, the His-94 substitution apparently affects the stability and/or the association of p22-*phox* with gp91-*phox* as well (de Boer et al. 1992a). In contrast, the Pro-156→Gln mutation in the C-terminal, hydrophilic part of p22-*phox* (patient 8, Table III) leaves the heme and the association with gp91-*phox* intact.

Altogether, nine different mutations have been found in eight A22 CGD families, indicating that this type of CGD is very heterogenous in nature. Moreover, only four polymorphisms have been recognized in the reading frame of p22-*phox* so far (Dinauer et al. 1990, de Boer et al. 1992a). Apparently, small changes in the structure of p22-*phox* already lead to instability and/or loss of function of this polypeptide.

MUTATIONS IN THE BETA SUBUNIT OF CYTOCHROME b_{558}

Deletions

The first 12 patients shown in Table IV suffer from X91 CGD caused by a deletion in the CYBB gene for gp91-*phox*. Although the size of these deletions varies widely, from about 5000 kilobases to single base pair deletions, this leads with only one exception to the occurrence of the $X91^0$ subtype of CGD. When the deletions are very large, not only the CYBB gene is affected, but neighboring genes as well. As a result, such patients suffer from other clinical syndromes in addition to CGD, e.g. Duchenne muscular dystrophy, retinitis pigmentosa and McLeods's syndrome (a mild hemolytic anemia with depressed levels of Kell antigens due to defects in the red-cell antigen K^x). This is the case in patients 1–4 (Table IV) (Kousseff 1981, Francke et al. 1985, Frey et al. 1988, de Saint-Basile et al. 1988).

Partial CYBB gene deletions have been found in several other patients (nrs. 5–8, Table IV). These include two brothers with two different deletions (patients 8.1 and 8.2), leading to deletion of exon 5 and exons 6 and 7, respectively. Analysis of their genomic DNA with restriction enzymes confirmed the size-analysis of the PCR-amplified cDNA. Sequencing of genomic DNA showed a very small overlap of the two deletions in intron V (de Boer and Roos, unpublished). Remarkably, the mother of these two brothers was found to carry both

TABLE IV
Summary of gp91-phox mutations in 51 patients with X91 CGD

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	Cytochrome b_{558} mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
1.	B.B.	M	X91^b	deletion	(0)	(0)	(0)	(0)	~5000 kb deletion	N.A.	Francke et al. 1985
2.	N.F.	M	X91^b	deletion	(0)	(0)	(0)	(0)	~4000 kb deletion	N.A.	Royer-Pokora et al. 1986
3.	O.M.	M	X91^0	deletion	0	0	0	0	~800 kb deletion	N.A.	Frey et al. 1988
4.	S.B.	M	X91^0	deletion	0	(0)	0	N.D.	N.D.	N.D.	de Saint-Basile et al. 1988
5.	T.S.	M	X91^0	deletion	N.D.	N.D.	N.D.	N.D.	~14 kb deletion	deletion of exons 4-9, frameshift	Roos 1993
6.	P.T.	M	X91^0	deletion	(0)	(0)	0	0	~10 kb deletion	N.R.	Pelham et al. 1990
7.	M.H.	M	X91^0	deletion	0	0	0	decreased	at least 6.5 kb deletion from exon 11-3' UT	deletion of exons 11-13	Zürich
8.1.	T.W.	M	X91^0	deletion	0	0	0	decreased	~3 kb deletion	deletion of exon 5	Roos 1993
8.2.	N.W.	M	X91^0	deletion	0	0	0	decreased	~3.5 kb deletion	deletion of exon 6+7	Roos 1993
9.	C.G.	M	X91^0	deletion	0	0	0	N.D.	TTC deletion after C-654	in frame deletion of Phe-215 or Phe-216	CLB, Amsterdam
10.	–	M	X91$^-$	deletion	~24%	21%	N.R.	N	AAG deletion after G-954	in-frame deletion of Lys-315	Curnutte 1993
11.	T.F.	M	X91^0	deletion	0	0	0	N.D.	T-59 deletion	frameshift, stop in codon 21	Roos 1993
12.	G.Q.	M	X91^0	deletion	0	0	0	N	T-134 deletion	frameshift, stop in codon 68	Roos 1993

TABLE IV
Continued

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	Cytochrome b_{558} mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
13.	–	M	X91^0	splice/deletion	0	0	0	N.D.	splice ag→aa at end of intron I	deletion exon 2 (in frame)	De Boer et al. 1992b
14.	–	M	X91^0	splice/deletion	0	0	N.R.	N	splice gt→tt at start of intron II	deletion exon 2 (in frame)	Curnutte 1993
15.	–	M	X91^0	splice/deletion	0	0	0	N.D.	splice ag→gg at end of intron II	deletion exon 3 (in frame)	Curnutte et al. 1993
16.	R.W.	M	X91^0	splice/deletion	0	0	0	decreased	splice gtaag→gtaaa at start of intron III	deletion exon 3 (in frame)	De Boer et al. 1992b

7.	M.H.	M	X91^0	deletion	0	0	0	decreased	~10 kb deletion at least 6.5 kb deletion from exon 11-3′ UT	N.R. deletion of exons 11-13	Pelham et al. 1990 Zürich
8.1.	T.W.	M	X91^0	deletion	0	0	0	decreased	~3 kb deletion	deletion of exon 5	Roos 1993
8.2.	N.W.	M	X91^0	deletion	0	0	0	decreased	~3.5 kb deletion	deletion of exon 6+7	Roos 1993
9.	C.G.	M	X91^0	deletion	0	0	0	N.D.	TTC deletion after C-654	in frame deletion of Phe-215 or Phe-216	CLB, Amsterdam
10.	–	M	X91$^-$	deletion	~24%	21%	N.R.	N	AAG deletion after G-954	in-frame deletion of Lys-315	Curnutte 1993
11.	T.R.	M	X91^0	deletion	0	0	0	N.D.	T-59 deletion	frameshift, stop in codon 21	Roos 1993
12.	G.Q.	M	X91^0	deletion	0	0	0	N	T-134 deletion	frameshift, stop in codon 60	Roos 1993

TABLE IV
Continued

					NADPH	Cytochrome b_{558}					
Nr.	Patient	Sex	CGD type	Mutation type	oxidase activity	protein	spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
13.	–	M	X91^0	splice/deletion	0	0	0	N.D.	splice ag→aa at end of intron I	deletion exon 2 (in frame)	De Boer et al. 1992b
14.	–	M	X91^0	splice/deletion	0	0	N.R.	N	splice gt→tt at start of intron II	deletion exon 2 (in frame)	Curnutte 1993
15.	–	M	X91^0	splice/deletion	0	0	0	N.D.	splice ag→gg at end of intron II	deletion exon 3 (in frame)	Curnutte et al. 1993
16.	R.W.	M	X91^0	splice/deletion	0	0	0	decreased	splice gtaag→gtaaa at start of intron III	deletion exon 3 (in frame)	De Boer et al. 1992b
17.	–	M	X91^0	splice/deletion	(0)	(0)	(0)	N.D.	splice gt→gc at start of intron V	deletion exon 5, frameshift, stop in codon 133	Curnutte et al. 1993
18.	D.D.	M	X91^0	splice/deletion	0	0	0	decreased, smaller	splice gta→gtt at start of intron V	deletion exon 5, frameshift, stop in codon 133	De Boer et al. 1992b
19.	B.S.	M	X91$^-$	splice/deletion	0	N.D.	~10%	N.D.	splice gtga deletion at start of intron VI	deletion exon 6, frameshift	Zürich
20.	R.H.	M	X91^0	"splice"/ deletion	0	0	0	N.D.	C-633→A	partial deletion exon 6, frameshift, stop in codon 206	De Boer et al. 1992b
21.	C.B.	M	X91^0	splice/deletion	0	0	0	decreased	splice gt→ga at start of intron VII	deletion exon 7, frameshift, stop in codon 230	De Boer et al. 1992b
22.	M.G.	M	X91$^-$	splice/deletion	6%	N	N	N	splice ag→gg at end of intron XI	deletion aa 488-497 in exon 12 (in frame)	Schapiro et al. 1991
23.	J.W.	M	X91^0	splice?/ deletion	0	0	0	0	~1 kb deletion from intron XII to 3′ UT	deletion C-terminal 41 aa (exon 13)	Royer-Pokora et al. 1986

TABLE IV
Continued

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	Cytochrome b_{558} mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
24.	R.C./ D.C.	2M	X91$^+$	missense	0	N	N	N	C-1256→A	Pro-415→His	Dinauer et al. 1989
25.	D.R.	M	X91$^+$	missense	0	N.D.	N	N.D.	C-1256→A	Pro-415→His	Zürich
26.	D.S.	M	X91$^+$	missense	0	N	N	N	A-1511→G	Asp-500→Gly	Leusen et al. 1994
27.	O.G.	M	X91$^-$	missense	0	N.D.	~30%	N.D.	C-170→A	Ala-53→Asp	Zürich
28.	H.K.R./ J.K.R.	2M	X91$^-$	missense	20-25%	decreased	~60%	N	C-179→T	Pro-56→Leu	CLB, Amsterdam
29.	R.L.	M	X91$^-$	missense	~5%	decreased Mr increased	~8%	N	G-478→A	Ala-156→Thr	Bolscher et al. 1991
30.	J.L.	M	X91$^-$	missense	5-10%	0	~40%	N	G-744→C	Cys-244→Ser	Bolscher et al. 1991
31.	D.H./ T.C.	2M	X91$^-$	missense	3-9%	< 10%	10-15%	N	G-937→A	Glu-309→Lys	Curnutte et al. 1993
32.	F.B.	M	X91$^-$	missense	10-20%	decreased Mr increased	~20%	N	G-1178→C	Gly-389→Ala	Bolscher et al. 1991
33.	–	M	X91^0	missense	0	0	N.R.	N.D.	G-70→C	Gly-20→Arg	Curnutte et al. 1993
34.	E.P.	F	X91^0	missense	(0)	(0)	(0)	N	A-314→G (heterozygous)	His-101→Arg	Bolscher et al. 1991
35.	P.B.	M	X91^0	missense	0	0	0	N	C-637→T	His-209→Tyr	Bolscher et al. 1991

TABLE IV
Continued

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	Cytochrome b_{558} mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
36.	M.Z.	M	X91^0	nonsense	0	0	0	N	T-111→A	Tyr-33→stop	CLB, Amsterdam
37.	B.C.	M	X91^0	nonsense	0	0	0	N	C-229→T	Arg-73→stop	Bolscher et al. 1991
38.	–	M	X91^0	nonsense	0	0	0	N.D.	C-283→T	Arg-91→stop	CLB, Amsterdam
39.	W.L.	M	X91^0	nonsense	0	0	0	N.D.	C-283→T	Arg-91→stop	Curnutte et al. 1993
40.	–	M	X91^0	nonsense	0	0	0	N.D.	C-481→T	Arg-157→stop	Curnutte et al. 1993
									C-688→T	Arg-226→stop	Curnutte 1993

[illegible]	[illegible]	[illegible]	[illegible]	missense	~5%	decreased Mr increased	~5%	N	G-478→A	Ala-156→Thr	Bolscher et al. 1991
30.	J.L.	M	X91⁻	missense	5-10%	0	~40%	N	G-744→C	Cys-244→Ser	Bolscher et al. 1991
31.	D.H./ T.C.	2M	X91⁻	missense	3-9%	<10%	10-15%	N	G-937→A	Glu-309→Lys	Curnutte et al. 1993
32.	E.B.	M	X91⁻	missense	10-20%	decreased Mr increased	~20%	N	G-1178→C	Gly-389→Ala	Bolscher et al. 1991
33.		M	X91⁰	missense	0	0	N.R.	N.D.	G-70→C	Gly-20→Arg	Curnutte et al. 1993
34.	E.P.	F	X91⁰	missense	(0)	(0)	(0)	N	A-314→G (heterozygous)	His-101→Arg	Bolscher et al. 1991
35.	P.B.	M	X91⁰	missense	0	0	0	N	C-637→T	His-209→Tyr	Bolscher et al. 1991

TABLE IV
Continued

Nr.	Patient	Sex	CGD type	Mutation type	NADPH oxidase activity	Cytochrome b_{558} protein	Cytochrome b_{558} spectrum	mRNA gp91-*phox*	Nucleotide change	Amino acid change	Reference
36.	M.Z.	M	X91⁰	nonsense	0	0	0	N	T-111→A	Tyr-33→stop	CLB, Amsterdam
37.	B.C.	M	X91⁰	nonsense	0	0	0	N	C-229→T	Arg-73→stop	Bolscher et al. 1991
38.	–	M	X91⁰	nonsense	0	0	0	N.D.	C-283→T	Arg-91→stop	CLB, Amsterdam
39.	W.L.	M	X91⁰	nonsense	0	0	0	N.D.	C-283→T	Arg-91→stop	Curnutte et al. 1993
40.	–	M	X91⁰	nonsense	0	0	0	N.D.	C-481→T	Arg-157→stop	Curnutte et al. 1993
41.	–	F	X91⁰	nonsense	(0)	(0)	N.R.	(0)	C-688→T (heterozygous)	Arg-226→stop	Curnutte 1993
42.	R.R.	M	X91⁰	nonsense	0	N.D.	0	N.D.	G-828→A	Trp-272→stop	Zürich
43.	–	M	X91⁰	nonsense	0	0	0	N.D.	C-880→T	Arg-290→stop	Curnutte et al. 1993
44.	J.M.	M	X91⁰	nonsense	0	0	0	N.D.	C-880→T	Arg-290→stop	Curnutte et al. 1993
45.	P.E.	M	X91⁰	insertion	0	0	0	decreased	insert 40 bp after G-702 in exon 7	13 additional aa after Gly-230, frameshift, stop in codon 253 (exon 7)	Rahbani et al. 1993
46.	–	M	X91⁰	insertion	0	0	0	low	insert G after G-207 in exon 3	frameshift, stop in exon 4	Curnutte et al. 1993
47.	–	M	X91⁰	insertion	0	0	N.R.	0	insert A between G-767 and T-773	frameshift, stop in exon 8	Curnutte 1993

0, zero; (0) presumed to be zero, judging from the mutation; N.A. not applicable; N.D., not determined; N.R., not reported; 3′ UT, 3′ untranslated mRNA region; Patients 8.1 and 8.2 are brothers, patients 24 are two brothers, patients 28 are also two brothers, and patients 31 are maternal first cousins. Patients 34 and 41 are female patiens with extreme lyonization; in these patients the control allele was found as well. Patients printed in bold were analyzed in our laboratory (CLB, Amsterdam). Zürich indicates patients who were analyzed in the Children's Hospital in Zürich, Switzerland (Prof. R. Seger, Dr. J. P. Hosale).

deletion alleles in her genomic DNA, as well as the normal allele. This family is now being studied in more detail.

Two patients have been found with triplet base-pair deletions that predict in-frame deletions of one amino acid (patients 9 and 10, Table IV). In one case, this led to an X91^0 CGD phenotype (patient 9), but in the other case, the cytochrome b_{558} expression and the NADPH oxidase activity showed a 20% residual level. Thus, patient 10 (Table IV) is a so-called 'variant' CGD patient with the X91$^-$ phenotype. Perhaps the Lys-315 deletion in this patient affects only the stability but not the function of the gp91-*phox* protein. Finally, 2 patients (11 and 12, Table IV) are known with single base-pair deletions, leading to decreased levels of mRNA for gp91-*phox* and frameshifts followed by premature termination of the gp91-*phox* translation. Because these deletions occurred early in the mRNA sequence, an X91^0 phenotype resulted.

Splice-site mutations

A common cause of X-linked CGD consists of splice site mutations (de Boer et al. 1992b). Table IV lists 11 patients with various forms of this aberration (patients 13–23). In patients 14,16,17,18,19 and 21 (Table IV) exon skipping during mRNA processing appeared to be due to single nucleotide substitutions in the donor splice sites of the relevant introns. In patients 13 and 15, missense mutations were found in the acceptor splice sites of introns I and II, respectively. As a result, the subsequent exons were skipped entirely during mRNA processing.

In patient 22 (Table IV), a similar mutation in the acceptor splice site of intron XI caused only partial skipping of exon 12, apparently because a cryptic splice site in this exon is activated. This results in skipping of only 30 nucleotides, predicting an in-frame deletion of 10 amino acids in the gp91-*phox* protein (Schapiro et al. 1991). According to the normal protein level on Western blot and the normal spectral characteristics of cytochrome b_{558}, this patient should be classified as an Xb$^+$ patient. However, according to the low NADPH oxidase activity of his neutrophils (about 6% of normal), this patient should be regarded as an Xb$^-$ CGD variant. Possibly, the 10 amino-acid deletion in the carboxyterminal domain of gp91-*phox* prohibits NADPH access to FAD in the activated cytochrome b_{558} molecule (Taylor et al. 1993).

The reverse situation exists in patient 20 (Table IV). In this patient, a mutation in exon 6 apparently creates a new splice site that is preferred over the normal donor splice site of intron VI. As a result, exon 6 is skipped from the site of the mutation to the 3′ end of the exon, which causes in addition a frameshift and a premature stop codon (de Boer et al. 1992b).

Finally, patient 23 (Table IV) lacks about 1 kilobase of his mRNA, resulting in deletion of exon 13 (the last exon) in the gp91-*phox* protein (Royer-Pokora et al. 1986). Probably, this is caused by a mutation in the acceptor splice site of intron XII. Because exon 13 con mRNA, the loss of this exon al phenotype in this patient.

In the other splice site patie Apparently, splice site mutations as extreme as in patient 23 (Tal and 22 (Table IV) show the Xb truncated proteins. Only in one the mRNA detectable on North

Thus, splice site mutations fr deletions of entire exons or exo of the disease.

Missense mutations

Missense mutations, leading t quently found in X-linked CG have no effect on mRNA sta cytochrome b_{558} in a variety of

Four patients from three dif non-functional cytochrome b_{55} brothers (case 24, Table IV) ca substitution (Dinauer et al. 198 P. Hossle et al., unpublished). B site of the cytochrome (Segal et from one of these patients wer azido-NADP. Indeed, labeling was strongly decreased as com al. 1992). Thus, the Pro-415→ gp91-*phox* protein or on its as the cytochrome non-functional

Another Xb$^+$ CGD patient patient (nr. 26, Table IV), an inhibition of p47-*phox* and p6 free activation system. To co around Asp-500 for docking c effect of a synthetic peptide co in this assay. Indeed, this pep and p67-*phox* to normal neutro oxidase activity in this system the structural model of cytoc which this domain of gp91-*ph*

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nino-acid deletion in the carboxyterminal
H access to FAD in the activated cyto-
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e site that is preferred over the normal
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h causes in addition a frameshift and a
92b).
out 1 kilobase of his mRNA, resulting
he gp91-*phox* protein (Royer-Pokora et
mutation in the acceptor splice site of

intron XII. Because exon 13 contains the 3′ untranslated region of the gp91-*phox* mRNA, the loss of this exon also causes mRNA instability, leading to an X91^0 phenotype in this patient.

In the other splice site patients, decreased amounts of mRNA were found. Apparently, splice site mutations always cause some mRNA instability, but never as extreme as in patient 23 (Table IV). Nevertheless, all patients except nrs. 19 and 22 (Table IV) show the Xb^0 phenotype, probably due to instability of the truncated proteins. Only in one patient (nr. 18, Table IV) was the smaller size of the mRNA detectable on Northern blot.

Thus, splice site mutations frequently occur in X-linked CGD and may cause deletions of entire exons or exon sections. In general, this leads to a severe form of the disease.

Missense mutations

Missense mutations, leading to single amino-acid replacements, are also frequently found in X-linked CGD (patients 24–35, Table IV). These mutations have no effect on mRNA stability, but affect the level and the function of cytochrome b_{558} in a variety of ways, leading to either Xb$^+$, Xb$^-$ or Xb^0 CGD.

Four patients from three different families are known with normal levels of non-functional cytochrome b_{558}, thus presenting with the Xb$^+$ phenotype. Two brothers (case 24, Table IV) carry point mutations that lead to a Pro-415→His substitution (Dinauer et al. 1989). A similar patient has been found in Zürich (J. P. Hossle et al., unpublished). Because Pro-415 is in the putative NADPH binding site of the cytochrome (Segal et al. 1992, Taylor et al. 1993), neutrophil membranes from one of these patients were tested for binding of the photo-affinity label 2-azido-NADP. Indeed, labeling at the position of gp91-*phox* (after SDS-PAGE) was strongly decreased as compared to normal neutrophil membranes (Segal et al. 1992). Thus, the Pro-415→His mutation has no effect on the stability of the gp91-*phox* protein or on its association with the p22-*phox* subunit, but renders the cytochrome non-functional by preventing NADPH binding.

Another Xb$^+$ CGD patient was recently investigated in our laboratory. In this patient (nr. 26, Table IV), an Asp-500→Gly mutation in gp91-*phox* causes total inhibition of p47-*phox* and p67-*phox* translocation to the membrane in the cell-free activation system. To confirm the importance of the gp91-*phox* domain around Asp-500 for docking of the cytosolic oxidase components, we tested the effect of a synthetic peptide corresponding to amino acids 491–504 of gp91-*phox* in this assay. Indeed, this peptide inhibited both the translocation of p47-*phox* and p67-*phox* to normal neutrophil membranes and the activation of the NADPH oxidase activity in this system (Leusen et al. 1994). These results perfectly fit with the structural model of cytochrome b_{558} constructed by Taylor et al. (1993), in which this domain of gp91-*phox* is supposed to prevent NADPH access to FAD

in the resting state of the cytochrome and to move away from the FAD cleft after activation by binding to p47-*phox* and/or p67-*phox*. Thus, also the Asp-500→Gly mutation has no effect on the stability of the gp91-*phox* protein or on its association with the p22-*phox* subunit, but renders cytochrome b_{559} non-functional by preventing activation of the cytochrome by p47-*phox* or p67-*phox*.

Eight patients from six different families (cases 27–32) were found with missense mutations that led to the variant Xb^- subtype of CGD. In these patients decreased amounts of gp91-*phox* and low NADPH oxidase activities were observed. Apparently, these mutations affect the stability of the gp91-*phox* protein or its association with the p22-*phox* subunit. As a result, the NADPH oxidase activity is decreased to a similar extent (Roos et al. 1992). In general, the mutations in these patients were found in the middle portion of gp91-*phox* and may have replaced amino acids involved in maintaining the secondary structure of the protein. These mutations are indicated in Fig. 4.

Finally, 3 patients have been detected with missense mutations leading to complete loss of gp91-*phox* expression, despite the presence of stable mRNA for this protein (nrs. 33–35, Table IV). One of these patients (nr. 34) is a female carrier of

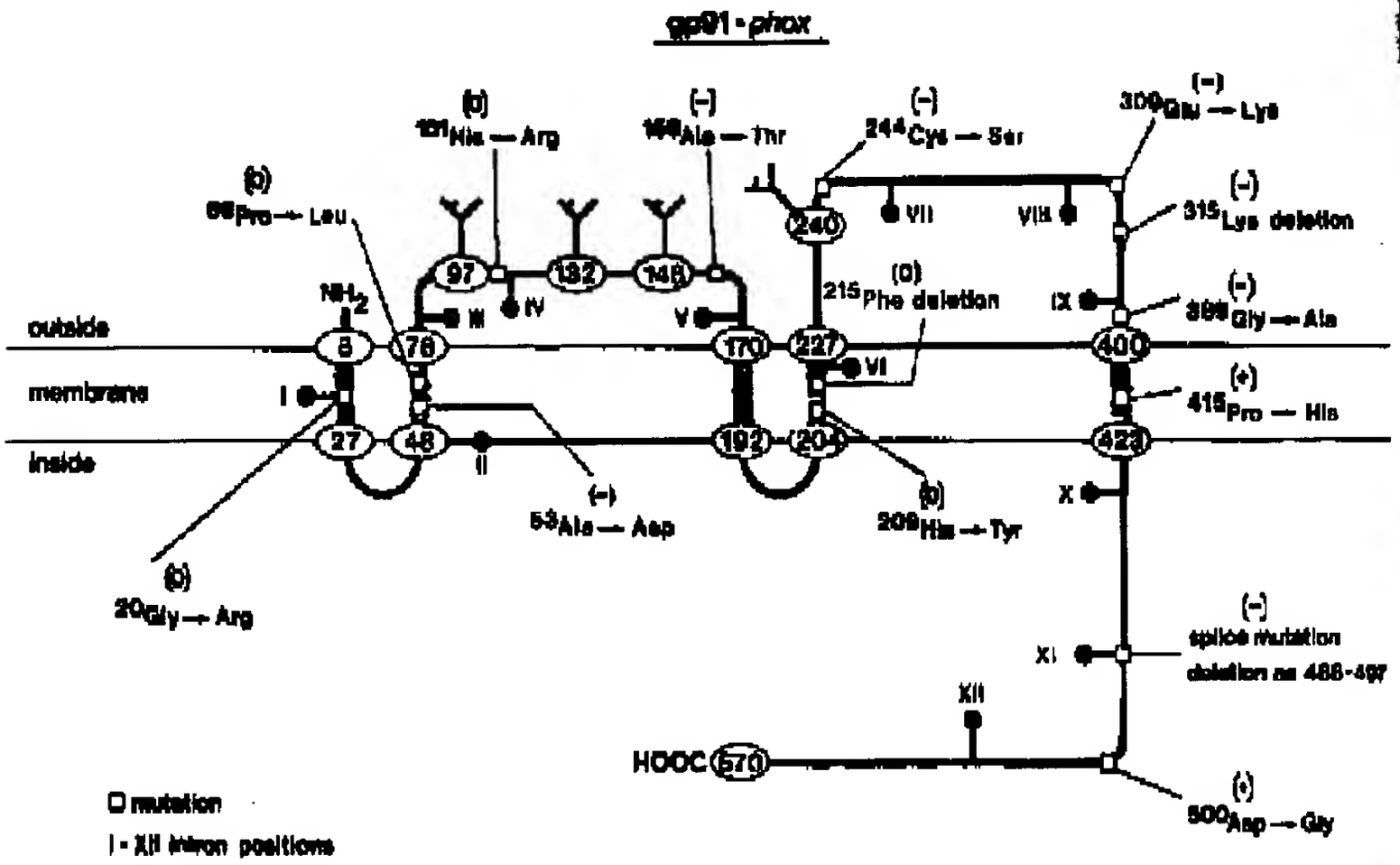


Figure 4. Schematic representation of gp91-*phox*. Indicated are the possible orientation of the peptide in the membrane (Imajoh-Ohmi et al. 1992), the N- and C-terminus, the intron positions (roman numerals), the possible glycosylation sites (Y) and the small mutations in the X91 CGD patients; (o) indicates $X91^0$, (−) indicates $X91^-$, (+) indicates $X91^+$ CGD.

X-linked CGD with an extreme l
In this patient, the control sequenc
with the mutated sequence (Bols
patients are either in the N-termin
hydrophobic stretches that might
histidyl residues that might be inv

Nonsense mutations

In 9 patients (36–45, Table IV), n
observed. Obviously, these muta
Remarkably, seven of these nine
the CGA codon for arginine in
female patient, heterozygous for
do not present with serious clin
inactivation may induce an unfi
for the mutated gp91-*phox* is ap

Insertions

The last type of mutations four
patients 46 and 47 (Table IV) s
predict premature termination o
of adenine cannot be localized
in the normal sequence at that
of Table III, in which a guanin
latter case, the six guanines we
neighboring cytosines.

In patient 45 (Table IV) we
exon 7 boundary (Rabbani et al.
caused by unequal crossing-o
predicted to be incorporated, f
termination of gp91-*phox* synth

All three insertions lead to
frameshifts – to the clinically s

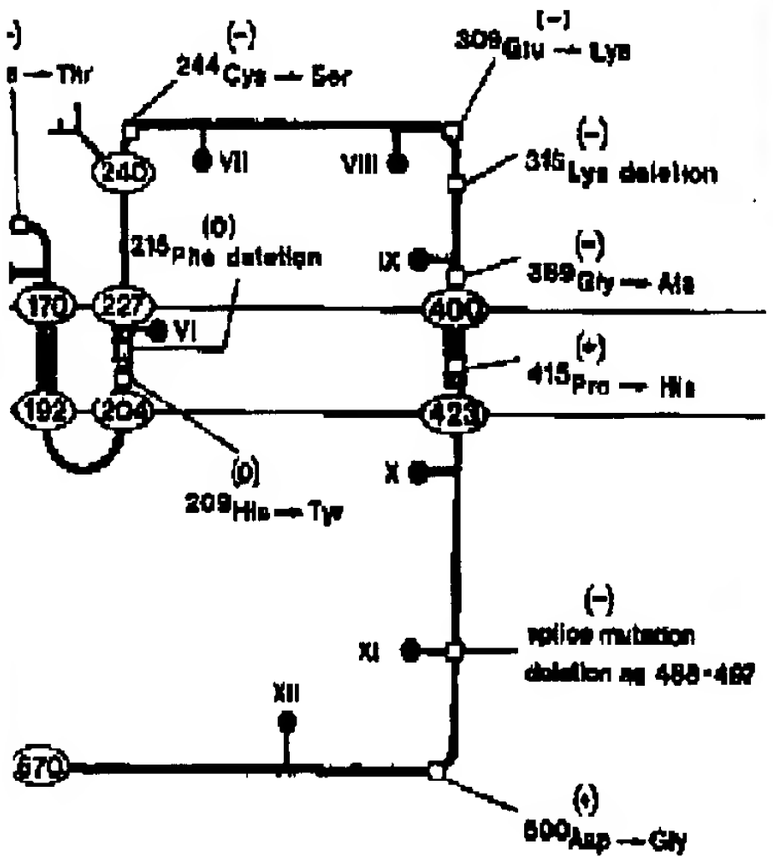
Other mutations

Finally, in 5 patients suspected
gp91-*phox* mRNA detectable
reverse transcriptase of the mR
with primers specific for gp91-

and to move away from the FAD cleft
x and/or p67-*phox*. Thus, also the Asp-
e stability of the gp91-*phox* protein or on
unit, but renders cytochrome b_{558} non-
he cytochrome by p47-*phox* or p67-*phox*.
les (cases 27–32) were found with missense
btype of CGD. In these patients decreased
oxidase activities were observed. Appar-
ty of the gp91-*phox* protein or its associ-
result, the NADPH oxidase activity is
. 1992). In general, the mutations in these
ion of gp91-*phox* and may have replaced
secondary structure of the protein. These

with missense mutations leading to com-
ite the presence of stable mRNA for this
ese patients (nr. 34) is a female carrier of

91-*phox*



ox. Indicated are the possible orientation of
al. 1992), the N- and C-terminus, the intron
osylation sites (Y) and the small mutations
, (−) indicates X91⁻, (+) indicates X91⁺

X-linked CGD with an extreme lyonization (2–5% positive cells in the NBT test). In this patient, the control sequence of gp91-*phox* cDNA was found in combination with the mutated sequence (Bolscher et al. 1991). The mutations in these last 3 patients are either in the N-terminal half of the protein, which contains most of the hydrophobic stretches that might serve as membrane-spanning regions, or remove histidyl residues that might be involved in heme binding (Fig. 4).

Nonsense mutations

In 9 patients (36–45, Table IV), nonsense mutations leading to a stop codon were observed. Obviously, these mutations all induced the $X91^0$ phenotype of CGD. Remarkably, seven of these nine mutations involved C→T substitutions, changing the CGA codon for arginine into the TGA stop codon. Patient 412 is another female patient, heterozygous for the mutation. Usually, carriers of X-linked CGD do not present with serious clinical problems, but non-random X-chromosome inactivation may induce an unfavorable phenotype. In this patient, the mRNA for the mutated gp91-*phox* is apparently unstable (Curnutte et al. 1993).

Insertions

The last type of mutations found in X-linked CGD is formed by insertions. In patients 46 and 47 (Table IV) single nucleotide insertions cause frameshifts and predict premature termination of gp91-*phox* synthesis. In patient 47, the insertion of adenine cannot be localized precisely, because five adenines are found already in the normal sequence at that point. A similar situation was found in patient 6 of Table III, in which a guanine is inserted in a strech of five guanines. In the latter case, the six guanines were probably stabilized by a hairpin-loop with six neighboring cytosines.

In patient 45 (Table IV) we found a 40-base-pair insertion at the intron VI/exon 7 boundary (Rabbani et al. 1993). This proved to be a 40-bp repeat, probably caused by unequal crossing-over. As a result, 13 additional amino acids are predicted to be incorporated, followed by 23 new amino acids and a premature termination of gp91-*phox* synthesis due to a frameshift.

All three insertions lead to decreased mRNA stability and – due to the frameshifts – to the clinically severe subtype of $X91^0$ CGD.

Other mutations

Finally, in 5 patients suspected of suffering from $X91^0$ CGD, we did not find gp91-*phox* mRNA detectable on Northern blot. Nevertheless, treatment with reverse transcriptase of the mRNA from these patients and amplification by PCR with primers specific for gp91-*phox* mRNA yielded fragments of the expected

size. However, the sequences of these products appeared normal (de Boer and Roos, unpublished). Therefore, in these patients, the disease may be due to the formation of unstable gp91-*phox* mRNA, for instance caused by mutations in the 3′ non-coding region. Alternatively, mutations in a promotor region may have led to decreased formation of gp91-*phox* mRNA. However, caution should be exercised when interpreting these results, because in 2 of these patients the X-linked nature of the disease was not proven (e.g. by a mosaic in the NBT test from an obligate carrier or by monocyte hybridization). Identification of the mutation in these last 5 patients awaits further analysis.

The list of different mutations leading to X-linked CGD clearly illustrates the very heterogeneous nature of these lesions. In fact, 44 different mutations were found in 46 families with this disease. Only patients 24 and 25, patients 38 and 39, and patients 43 and 44, have the same mutations. Because polymorphisms within the coding region of the CYBB gene are not known, it appears that the gp91-*phox* polypeptide is extremely sensitive to mutations.

MUTATIONS IN CYTOSOLIC NADPH OXIDASE COMPONENTS

Mutations in p47-phox

In contrast to the large heterogeneity found in A22 and X91 CGD, only four different mutations are known so far to cause A47 CGD. In 10 unrelated CGD patients with p47-*phox* deficiency, a dinucleotide deletion was found at a GTGT tandem repeat, corresponding to the first four bases of exon 2 (Casimir et al. 1991, Chanock et al. 1991, Volpp & Lin 1993). Six patients have a homozygous GT deletion, which results in a frameshift and premature translation termination after the synthesis of a 50-amino-acid protein. The other 4 patients are compound heterozygotes for this GT deletion in combination with point mutations, i.e. A-179→G predicting Thr-53→Ala substitution, A-425→G leading to Lys-135→Glu replacement, or G-502 deletion predicting a frameshift and premature stop codon. In our own laboratory, we have analyzed the cDNA of 17 $A47^0$ CGD patients. In all cases, the GT deletion was found, without other point mutations or deletions (de Boer and Roos, unpublished).

In all patients, the mRNA for p47-*phox* is present in apparently normal amounts and with a normal size, as judged from Northern blots with mRNA isolated from mononuclear leukocytes (Lomax et al. 1989, Casimir et al. 1991, Chanock et al. 1991, de Boer and Roos, unpublished). In contrast, p47-*phox* (or a truncated derivative) is always undetectable in neutrophil lysates. Thus, it appears that all four mutations lead to the synthesis of an unstable protein.

A large number of polymorphisms have been detected in the NCF-1 gene, some of them predicting incorporation of different amino acids (S. Chanock, pers. commun.). Hence, the p47-*phox* protein is less dependent on a critical conformation for its function than the cytochrome b_{558} subunits.

Mutations in p67-phox

A similar situation exists in A67 C
mRNA for p67-*phox* but no pro
Recently, we have located the mut
to be homozygous for a G-233→
78→Glu replacement. Both paren
for this mutation, although the pa
(de Boer et al. 1994). In another A
in the mRNA but not in the ge
suspected, but has not yet been cl
In a 3rd A67→patient, a GAA de
It is not yet known whether this
(de Klein and Roos, unpublished
to be larger than that in p47-*pho*

Diagnosis

In a patient with clinical sympto
confirmed by the hallmark of C
increased NADPH oxidase activi
The oxidase activity can be mea
electrode), superoxide generation
of hydrogen peroxide (oxidation
Roos et al. 1983). Chemiluminesc
to measure oxidase activity (W
methods have been introduced f
Stimuli frequently used to activat
an and phorbol-myristate acetat
blood can also be used (Roos et

Differentiation between the fo
analysis of neutrophil lysates wi
phox and p67-*phox*. In case of A
lack of reactivity with the relev
+ or – variants of these subgrou
CGD, however, the distinction
cytochrome b_{558} are absent in A
Verhoeven et al. 1989) and + a
1989, 1991, Schapiro et al. 199
both subunits of cytochrome b_5

products appeared normal (de Boer and
e patients, the disease may be due to the
NA, for instance caused by mutations in
y, mutations in a promoter region may
1-*phox* mRNA. However, caution should
ults, because in 2 of these patients the X-
roven (e.g. by a mosaic in the NBT test
yte hybridization). Identification of the
further analysis.
g to X-linked CGD clearly illustrates the
ons. In fact, 44 different mutations were
Only patients 24 and 25, patients 38 and
ame mutations. Because polymorphisms
gene are not known, it appears that the
itive to mutations.

ADPH OXIDASE COMPONENTS

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cause A47 CGD. In 10 unrelated CGD
cleotide deletion was found at a GTGT
st four bases of exon 2 (Casimir et al.
1993). Six patients have a homozygous
t and premature translation termination
tein. The other 4 patients are compound
mbination with point mutations, i.e. A-
ion, A-425→G leading to Lys-135→Glu
a frameshift and premature stop codon.
d the cDNA of 17 A47^0 CGD patients.
ithout other point mutations or deletions

phox is present in apparently normal
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omax et al. 1989, Casimir et al. 1991,
unpublished). In contrast, p47-*phox* (o
ectable in neutrophil lysates. Thus, it
e synthesis of an unstable protein.
ve been detected in the NCF-1 gene,
of different amino acids (S. Chanock,
rotein is less dependent on a critical
tochrome b_{558} subunits

Mutations in p67-phox

A similar situation exists in A67 CGD: all patients analyzed so far have normal mRNA for p67-*phox* but no protein (Leto et al. 1990, de Boer et al. 1994). Recently, we have located the mutation in one A67^0 CGD patient, who appeared to be homozygous for a G-233→A substitution. This mutation predicts a Gly-78→Glu replacement. Both parents and a sister of the patient are heterozygotes for this mutation, although the parents are not known to be related to each other (de Boer et al. 1994). In another A67^0 patient, we have found an exon 3 deletion in the mRNA but not in the genomic DNA. Hence, a splice site mutation is suspected, but has not yet been characterized (de Klein and Roos, unpublished). In a 3rd A67→patient, a GAA deletion was found, predicting a Lys-58 deletion. It is not yet known whether this is a homozygous or a heterozygous mutation (de Klein and Roos, unpublished). Thus, the heterogeneity in p67-*phox* appears to be larger than that in p47-*phox*.

DIAGNOSIS AND TREATMENT OF CGD

Diagnosis

In a patient with clinical symptoms suggestive of CGD, the diagnosis has to be confirmed by the hallmark of CGD: failure of the neutrophils to react with increased NADPH oxidase activity upon treatment with an appropriate stimulus. The oxidase activity can be measured by oxygen consumption (with an oxygen electrode), superoxide generation (reduction of ferri-cytochrome c) or production of hydrogen peroxide (oxidation of homovanillic acid) (Weening et al. 1974, 1975, Roos et al. 1983). Chemiluminescence with luminol or lucigenin is also often used to measure oxidase activity (Weening et al. 1985b). Recently, flowcytometric methods have been introduced for the diagnosis of CGD (Roesler et al. 1991). Stimuli frequently used to activate the NADPH oxidase are serum-treated zymosan and phorbol-myristate acetate. The neutrophils are usually purified, but full blood can also be used (Roos et al., unpublished).

Differentiation between the four subgroups of CGD begins with Western blot analysis of neutrophil lysates with antibodies against p22-*phox*, gp91-*phox*, p47-*phox* and p67-*phox*. In case of A47 or A67 CGD, the distinction is easy, because lack of reactivity with the relevant antibodies is the rule, but the possibility of + or – variants of these subgroups must be kept in mind. In case of A22 or X91 CGD, however, the distinction can be more difficult, because both subunits of cytochrome b_{558} are absent in A22^0 as well as in X91^0 CGD (Parkos et al. 1989, Verhoeven et al. 1989) and + and – variants are known to exist (Dinauer et al. 1989, 1991, Schapiro et al. 1991, Roos et al. 1992, Leusen et al. 1994). When both subunits of cytochrome b_{558} are undetectable, distinction between A22 and

X91 CGD can usually be made by searching for carriers in the family of the patients with the NBT slide test (see next paragraph). The presence of neutrophils with functional and neutrophils with non-functional NADPH oxidase in obligate heterozygotes (e.g. the mothers of the patients) proves the X-linked nature of the disease, and thus points to a deficiency in gp91-*phox*. Of course, if the patient is female, this in itself is an indication that the disease probably has an autosomal origin, and hence may be caused by a deficiency in p22-*phox*, but it must be kept in mind that extreme lyonization in carriers of gp91-*phox* deficiency may lead to clinical problems as well. When both subunits of cytochrome b_{558} are detectable on protein blots with the appropriate antibodies, a (relative) deficiency of NADPH oxidase activity of the patient's neutrophil membranes in the cell-free system will prove a defect in cytochrome b_{558}. In that case, analysis of family members with the NBT slide test is again indicated.

Carrier detection in the X91 subtype of CGD is based on detection of functional and non-functional individual cells. This can be performed with the NBT slide test, in which neutrophils are incubated with the pale yellow dye nitro tetrazolium (NBT), activated (e.g. with phorbol-myristate acetate) and scored microscopically for deposits of black formazan (NBT reduced by superoxide) (Meerhof & Roos 1986). A mosaic of stained and non-stained cells proves the carrier state of X91 CGD. Similar assays are possible with flowcytometric methods (Mizuno et al. 1988, Roesler et al. 1991). However, about one-third of all X-linked defects arises from new mutations in germ-line cells. Moreover, extreme lyonization towards the normal phenotype may obscure the detection of X91 CGD carriers. Therefore, failure to detect these carriers does not disprove the X-linked origin of the disease.

Carriers of the autosomal subtypes of CGD are less easy to recognize. Even in the neutrophils from obligate heterozygotes, no abnormalities in any of the NADPH oxidase activity assays can be detected. However, we have found that oxygen consumption and superoxide production of these cells after activation with phorbol-myristate acetate is significantly lower than that of normal neutrophils. This gene-dose effect is detectable in carriers of $A47^0$ CGD (Verhoeven et al. 1988) as well as in carriers of $A67^0$ CGD (de Boer et al. 1993), but has not yet been tested in carriers of $A22^0$ or $A22^+$ CGD. Of course, when the mutation in a patient is known, carriers among family members of any CGD subtype can easily be recognized at the DNA level.

Prenatal diagnosis

Before the NADPH oxidase components had been cloned, prenatal diagnosis of CGD could only be performed by analysis of umbilical blood phagocytes, e.g. with the NBT slide test or with a whole-blood oxygen consumption assay (Newburger et al. 1979). However, fetal blood samples cannot be obtained before

16–18 weeks gestation. This
affected fetusses. With the av
cytic cells are no longer requir
oxidase components. Either R
or detection of specific gene
biopsy or amniocentesis can pr
at risk. Most efforts in this resp
sons of carriers of this disease
of who the father is.

In case of a complete or p
will suffice to identify patient
this technique has been emplo
was unaffected (Orkin 1989).
not have DNA abnormalities
RFLPs within the CYBB gen
have now been recognized (Ba
et al. 1990, Francke et al. 19
families to whom first-trimes
three regions with a variable
the CYBB gene (Gorlin 1991
region, due to allelic differenc
increasing the reliability of R

Of course, if the specific,
diagnosis becomes relatively s
the CGD status of a subseque
(de Boer et al. 1992c). Link
confirmed this diagnosis wit
pregnancy was terminated a
blood cells by lack of oxyg
Subsequently, this method of
(de Boer and Roos, unpublis
lished as point mutations in
Table IV). Subsequently, the
origin and found to be norm
diagnoses.

Within the NCF-2 gene, c
*Hin*dIII (Kenney & Leto 199
in which a patient with A67
patient and her mother were
zygous. Fetal DNA, obtained
tation and grown for 3 week
RFLP as well, indicating tha

arching for carriers in the family of the
t paragraph). The presence of neutrophils
n-functional NADPH oxidase in obligate
atients) proves the X-linked nature of the
in gp91-*phox*. Of course, if the patient is
at the disease probably has an autosomal
eficiency in p22-*phox*, but it must be kept
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ubunits of cytochrome b_{558} are detectable
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in indicated.
: of CGD is based on detection of func-
cells. This can be performed with the
are incubated with the pale yellow dye
g. with phorbol-myristate acetate) and
of black formazan (NBT reduced by
A mosaic of stained and non-stained
CGD. Similar assays are possible with
. 1988, Roesler et al. 1991). However,
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origin of the disease.
f CGD are less easy to recognize. Even
ygotes, no abnormalities in any of the
detected. However, we have found that
oduction of these cells after activation
antly lower than that of normal neutro-
in carriers of A47^0 CGD (Verhoeven et
CGD (de Boer et al. 1993), but has not
$^+$ CGD. Of course, when the mutation
nily members of any CGD subtype can

had been cloned, prenatal diagnosis of
sis of umbilical blood phagocytes, e.g.
lood oxygen consumption assay (New-
d samples cannot be obtained before

16–18 weeks gestation. This means second-trimester abortions for carriers of affected fetusses. With the availability of molecular-biology techniques, phagocytic cells are no longer required for the detection of genetic defects in NADPH oxidase components. Either RFLPs (restriction fragment length polymorphisms) or detection of specific gene defects in fetal DNA obtained by chorionic villus biopsy or amniocentesis can provide the means for a definite diagnosis for families at risk. Most efforts in this respect have been directed towards X91 CGD, because sons of carriers of this disease have a 50% chance of being patients, irrespective of who the father is.

In case of a complete or partial gene deletion, simple Southern blot analysis will suffice to identify patients. Indeed, in the family of patient 23 (Table IV), this technique has been employed to demonstrate that a subsequent male fetus was unaffected (Orkin 1989). However, most families at risk for X91 CGD do not have DNA abnormalities that are detectable in this manner. Fortunately, two RFLPs within the CYBB gene after digestion with the restriction enzyme *Nsi*I have now been recognized (Battat & Francke 1989, Pelham et al. 1990, Mühlebach et al. 1990, Francke et al. 1990b), increasing to about 50% the proportion of families to whom first-trimester prenatal diagnosis can be offered. Moreover, three regions with a variable number of tandem repeats (VNTRs) are present in the CYBB gene (Gorlin 1991). It is to be expected that polymorphism at this region, due to allelic differences in the number of repeats, can be used for further increasing the reliability of RFLP-based X91 CGD detection.

Of course, if the specific, family-based mutation can be identified, prenatal diagnosis becomes relatively simple. Recently, we have demonstrated in this way the CGD status of a subsequent male fetus in the family of patient 16 (Table IV) (de Boer et al. 1992c). Linkage studies with RFLPs around the CYBB locus confirmed this diagnosis with >98% reliability. On request of the family, the pregnancy was terminated at week 15. The diagnosis was confirmed on fetal blood cells by lack of oxygen consumption and a negative NBT slide test. Subsequently, this method of prenatal diagnosis was used in 2 additional cases (de Boer and Roos, unpublished). In both families, the mutation was first established as point mutations in the coding sequence of CYBB (patients 11 and 37, Table IV). Subsequently, the chorionic DNA was analyzed, checked for fetal origin and found to be normal in both cases. Linkage studies confirmed these diagnoses.

Within the NCF-2 gene, one RFLP has been discovered after digestion with *Hin*dIII (Kenney & Leto 1990). This has been used to analyze a fetus in a family in which a patient with A67^0 CGD had been previously born. This proband patient and her mother were homozygous for this RFLP; the father was heterozygous. Fetal DNA, obtained from amnionic fibroblasts taken at 12 weeks gestation and grown for 3 weeks, showed the fetus to be a heterozygote for this RFLP as well, indicating that the fetus had received a normal allele from the

father (Kenney et al. 1993). The baby was carried to term, and a boy was born who was shown to have a normal phenotype.

Treatment

Until recently, the major approach to treatment of CGD patients was aimed at prevention and aggressive treatment of infections. Prevention includes routine immunizations, prompt cleaning and antiseptic treatment of skin wounds, careful anal and dental hygiene, abstinence from smoking and avoidance of contact with decaying plant material that may contain *Aspergillus* spores (Smith & Curnutte 1991). The use of prophylactic antibiotics, especially sulphamethoxazole-trimethoprim, is very effective (Weening et al. 1983, Callin et al. 1983, Mouy et al. 1989, Margolis et al. 1990). The use of anti-fungal agents, e.g. itraconazole, may be indicated (Fischer et al. 1993). Treatment includes prompt surgical drainage of abscesses and early and prolonged use of systemic antimicrobials. The use of daily white blood cell transfusions in life-threatening situations has also been advocated (Gallin et al. 1983). Allogeneic bone marrow transplantation has been attempted, but with little success due to severe transplantation complications (Rappeport et al. 1982, Kamani et al. 1988). Perhaps the use of antibodies against LFA-1 (CD11a), to inhibit graft-versus-host disease, will improve future bone-marrow transplantation results in CGD patients (Fischer et al. 1991).

The latest development in the treatment of CGD has been the use of interferon-γ (IFN-γ). First, it was proven that addition of IFN-γ *in vitro* enhanced both the superoxide production and the level of mRNA for gp91-*phox* of normal phagocytes (Cassatella et al. 1985, Berton et al. 1986). Thereafter, neutrophils and monocytes from X91^0, X91$^-$ and A47^0 CGD patients were treated with IFN-γ *in vitro*. Cells from X91^0 CGD patients did not respond, but those from X91$^-$ and A47^0 CGD patients did (Ezekowitz et al. 1987, Sechler et al. 1988, Weening et al. 1988). Based on these findings, two small groups of CGD patients were treated with subcutaneous injections of IFN-γ (Sechler et al. 1988, Ezekowitz et al. 1988). In general, the same phenomena were noted: a large increase in O_2^- generating capacity and killing of *Staph. aureus in vitro*, and modest increase in heme signal and mRNA for gp91-*phox* in Xb$^-$ patients. All A47^0 patients responded, but to a limited degree. Of the X91^0 patients, only a few responded with a partial restoration of functions. Given the fact that many of the X91^0 patients will suffer from gene deletions and translation termination mutations, this last result is not surprising.

However, these limited studies did not involve enough patients to evaluate any clinical benefits of IFN-γ. Therefore, a large multicenter study has been carried out, in which 128 CGD patients were enrolled (Int. Chronic Granulomatous Disease Cooperative Study Group, 1991). The patients were randomized according to sex, use of prophylactic antibiotics, genetic background

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double-blinded. The results show
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Gene therapy

Because CGD is a disorder of
defects, transfer of the correct ge
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therapy. The genetically enginee
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Carriers for X91^0 CGD with les
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An important step forewards
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oup, 1991). The patients were ran-
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of their disease and treatment center. The study was placebo-controlled and double-blinded. The results showed that recombinant human IFN-γ, given in a dose of 0.05 mg/m^2 subcutaneously three times a week, caused a 70% reduction in the incidence of serious infections (requiring hospitalization and the use of parenteral antibiotics), regardless of the type of CGD. In contrast to the earlier reports, however, most patients in this larger study showed no significant improvement in O_2^- production or bacterial killing by their neutrophils *in vitro*. Thus, rhIFN-γ appears to boost host defense by other mechanisms, e.g. by augmentation of non-oxidative mechanisms and/or improvement of diapedesis and locomotion.

Gene therapy

Because CGD is a disorder of marrow-derived cells with well-defined genetic defects, transfer of the correct gene for the defective NADPH oxidase component into pluripotent hemopoietic stem cells would, in principle, constitute definitive therapy. The genetically engineered stem cells can then be returned to the bone marrow of a patient, with subsequent production of corrected mature phagocytes. Carriers for X91^0 CGD with less than 10% of normal cells may have a normal phenotype (Roos et al. 1986), suggesting that correction of only a small percentage of the cells in CGD patients will result in a clinical improvement or cure. Recent studies from several laboratories have demonstrated that p47-*phox* protein expression and NADPH oxidase activity can be partially restored in EBV-transformed B-lymphocyte lines established from A47^0 CGD patients after transduction or transfection with retrovirus or other expression vectors containing p47-*phox* cDNA (Cobbs et al. 1992, Thrasher et al. 1992, Chanock et al. 1992, Volpp & Lin 1993). In addition, transfection of EBV B-cell lines from X91^0 CGD patients with a vector containing gp91-*phox* cDNA has been reported to partially correct gp91-*phox* protein expression and NADPH oxidase activity (Porter et al. 1993). However, EBV-transformed lymphocytes are not relevant targets for gene therapy of CGD, because these cells are different from the myelomonocytic cells that are deficient in CGD.

An important step forewards, therefore, was the recent publication by Sekhsaria et al. (1993), who reported transfection of peripheral blood hematopoietic progenitor cells with a retroviral vector containing p47-*phox* cDNA. When progenitor cells from A47^0 patients were used, this procedure resulted in efficient correction of NADPH oxidase activity when these cells were differentiated *in vitro* to mature neutrophils and monocytes. It remains to be proven that such transfected progenitor cells will sufficiently reconstitute the bone marrow of A47^0 CGD cells to cure the patients. In addition, transcription of DNA sequences for gp91-*phox* has been shown to require *cis* elements and *trans* factors that have not yet been fully elucidated (Skalnik et al. 1991b). Hence, a genetic cure for X91

CGD patients may prove to be more difficult than for A47 CGD patients. Nevertheless, gene therapy for CGD patients may be expected in the not-too-distant future.

SUMMARY

Chronic granulomatous disease is a serious clinical entity. The disease is caused by the failure of NADPH oxidase in phagocytic leukocytes to generate superoxide, needed for the killing of micro-organisms. The patients need careful management aimed at prevention and aggressive treatment of infections. CGD is a heterogeneous syndrome, both clinically and genetically. This disease is caused by a diversity of mutations, and multiple genes are affected. In fact, in the A22 and X91 subtypes of CGD, in which the alpha subunit and the beta subunit of cytochrome b_{558} are affected, respectively, the mutations are virtually unique for each CGD family tested. The results of these studies provide a better understanding of the mechanism of action of the various components of the superoxide-generating enzyme. Although treatment of CGD patients has improved considerably over the past 30 years, death caused by overwhelming infections is still a serious threat. Prenatal diagnosis now provides the relatives of a CGD patient with the possibility to choose for first-trimester abortion of an affected fetus. Moreover, genetic correction of the disease is now a goal within reach.

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EDGMENTS

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